

University of Groningen

## Honey, I Shrunk the DNA

Oijen, Antoine M. van

*Published in:*  
Biopolymers

*DOI:*  
[10.1002/bip.20624](https://doi.org/10.1002/bip.20624)

**IMPORTANT NOTE:** You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2007

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*

Oijen, A. M. V. (2007). Honey, I Shrunk the DNA: DNA Length as a Probe for Nucleic-Acid Enzyme Activity. *Biopolymers*, 85(2), 144-153. <https://doi.org/10.1002/bip.20624>

**Copyright**

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

**Take-down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

*Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.*

## Review

# Honey, I Shrunk the DNA: DNA Length as a Probe for Nucleic-Acid Enzyme Activity

Antoine M. van Oijen

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115

Received 29 September 2006; accepted 31 October 2006

Published online 2 November 2006 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/bip.20624

### ABSTRACT:

The replication, recombination, and repair of DNA are processes essential for the maintenance of genomic information and require the activity of numerous enzymes that catalyze the polymerization or digestion of DNA. This review will discuss how differences in elastic properties between single- and double-stranded DNA can be used as a probe to study the dynamics of these enzymes at the single-molecule level. © 2006 Wiley Periodicals, Inc. *Biopolymers* 85:144–153, 2007.

**Keywords:** single-molecule; DNA; polymerase; nuclease; replication

This article was originally published online as an accepted preprint. The "Published Online" date corresponds to the preprint version. You can request a copy of the preprint by emailing the *Biopolymers* editorial office at [biopolymers@wiley.com](mailto:biopolymers@wiley.com)

### INTRODUCTION

A wide variety of structural and biochemical techniques are available to study the mechanistic principles underlying the enzymatic catalysis of chemical reactions. The addition of imaging and molecular manipulation techniques to the toolbox of biophysical chemistry has enabled researchers to study the behavior of individual molecules. The ability to record "molecular

movies" of enzymes at the single-molecule level, and thus remove averaging over large numbers of molecules participating in the reaction, has provided unique insights into their dynamics and reaction mechanisms. These developments have led to a greater understanding of biological processes which include, but are not limited to, active cellular transport,<sup>1–6</sup> muscle contraction,<sup>7–11</sup> ion transport,<sup>12</sup> ATP synthesis,<sup>13–15</sup> and redox reactions.<sup>16</sup>

A field of significant activity within the single-molecule community is the study of processes that act upon DNA. The robustness of DNA as a substrate and the development of techniques to manipulate individual DNA molecules<sup>17</sup> have allowed a large number of nucleic-acid enzymes to be characterized at the single-molecule level. The results of these studies have contributed to new insights in the areas of transcription,<sup>18–23</sup> recombination,<sup>24–30</sup> repair,<sup>31</sup> and replication.<sup>32–37</sup> The physical properties of DNA itself have been thoroughly characterized by stretching individual DNA molecules and monitoring their length as a function of parameters such as force and torque.<sup>38</sup> In this review, I will discuss how changes in the physical properties of DNA can be exploited to study the dynamics of nucleic-acid enzymes at the single-molecule level.

### MEASURING ELASTIC PROPERTIES OF DNA

The recent development of methods to mechanically manipulate single DNA molecules has led to a detailed understanding of the mechanical properties of DNA.<sup>38</sup> The controlled stretching of individual DNA molecules allowed researchers to quantitatively describe the mechanical properties of double-stranded DNA in terms of theoretical models originally developed to describe the behavior of ideal polymers.<sup>39,40</sup> The following section will discuss the differences in elastic properties between single- and double-stranded DNA and how they can be used to study the activity of nucleic-acid enzymes at the single-molecule level.

Correspondence to: Antoine M. van Oijen; e-mail: [antoine\\_van\\_oijen@hms.harvard.edu](mailto:antoine_van_oijen@hms.harvard.edu)



© 2006 Wiley Periodicals, Inc.

## Mechanical Manipulation of Individual DNA Molecules

By immobilizing one end of a linear DNA molecule and exerting a force on the other end, the molecule can be stretched and its extension studied under a variety of physical and biochemical conditions. Typically, the force is applied on the DNA through a small bead attached to the free end of the molecule. Force is exerted on the bead by laminar flow, a magnetic field, or optical trapping.

**Flow Stretching.** A laminar flow of aqueous buffer can be used to exert forces on objects by transfer of momentum from the fluid to the object. DNA can be attached on one end to the surface of a flow chamber and on the other end to a polystyrene bead whose viscous drag exerts a force on the DNA.<sup>41–43</sup> Combining the flow stretching with low-magnification wide-field optical microscopy allows for a simultaneous observation of multiple bead-DNA tethers. This multiplexing is essential for gathering statistically significant sample sizes and for observing the low-probability events associated with the activity of complex, multiprotein machineries. A drawback of flow stretching is that forces cannot be changed rapidly. Furthermore, residual instabilities in flow velocities result in force and spatial resolutions not as high as can be achieved with optical or magnetic trapping.

**Magnetic Tweezers.** Magnetic fields can be used to manipulate and apply forces to DNA or proteins that are tethered to paramagnetic beads, with the unique advantage that the presence of a preferred magnetization axis in the magnetic beads allows them to be aligned with the direction of the applied magnetic field.<sup>44–46</sup> This method enables individual DNA molecules, bound to a surface on one end and to a magnetic bead to the other, to be over- or underwound, and thus supercoiling to be introduced. The length of the vertically stretched DNA is typically obtained by monitoring the transversal Brownian motion of the bead or by analyzing its Airy diffraction pattern. The resultant requirement for high-resolution imaging (either temporally or spatially) prevents the tracking of many beads simultaneously.

**Optical Trapping.** Optical tweezers exploit the fact that light exerts force on matter. Dielectric particles, such as polystyrene beads or bacteria, are attracted to the center of a tightly focused laser beam and can be trapped there.<sup>47,48</sup> The force exerted on the object depends on the power of the laser, the dimensions of the object, and the difference in index of refraction between the object and the surrounding medium. The large range in forces that can be applied (100 fN–100 pN) makes optical trapping techniques suitable for the

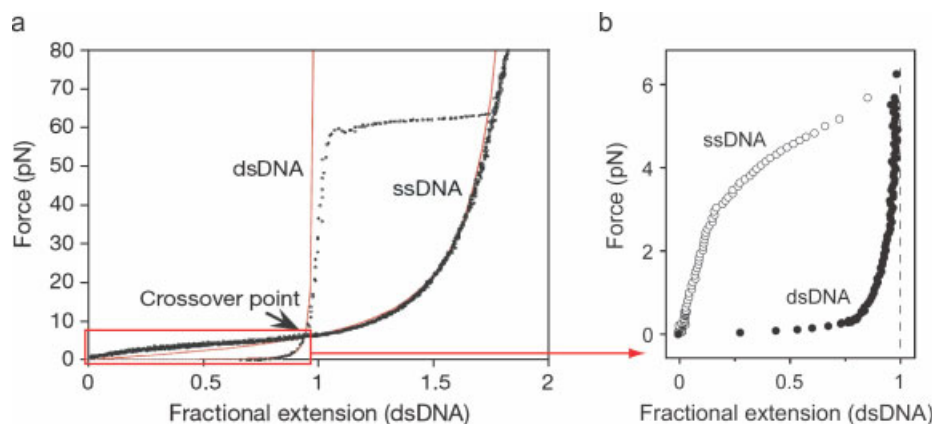
investigation of the effect of force on biochemical processes.<sup>49</sup> The high spatial accuracy associated with optical trapping has enabled measurements of translocating nucleic-acid enzymes with a resolution of a single base pair.<sup>19</sup> Generally, only a small number of beads can be trapped simultaneously, making multiplexed observations difficult.

## Elastic Properties of DNA

The relation between the extension of individual DNA molecules and applied force can be characterized over a large range of forces. Figure 1 shows force-extension data of individual molecules of single- and double-stranded  $\lambda$  phage DNA. Theoretical models developed to describe the elastic properties of polymers have been successfully applied to rationalize these results. The relation between force and extension of double-stranded DNA can be described by the worm-like chain model (WLC), which characterizes a polymer using a single parameter: the bending persistence length. The WLC model treats the DNA as a flexible rod that displays smooth, random bends as a result of thermal fluctuations. The local direction of the rod loses correlation on length scales larger than the bend persistence length. Double-stranded DNA is considerably stiffer than single-stranded DNA, as indicated by their different persistence lengths,  $\sim 50$  nm for duplex DNA<sup>39,51</sup> and 0.8–3 nm for the single-stranded form (with a strong dependence on the ionic strength of the solution).<sup>50,52</sup>

The energy required to bend a stretch of DNA of a certain length over a certain angle is linearly dependent on the bending persistence length: the shorter the persistence length, the less energy required to bend. As a consequence, a polymer with a short bending persistence length will adopt a more compact random-coil structure with an average end-to-end distance much shorter than its contour length. Extending the molecule is entropically unfavorable because the number of possible configurations decreases as the molecule is stretched out. For very small stretching forces, thermal fluctuations will prevent the DNA from being extended. The ratio of the thermal energy ( $k_B T$ ) to the persistence length determines the force needed to overcome thermal fluctuations and increase the molecule's end-to-end distance. The difference in persistence length between single- and double-stranded DNA results in a difference of forces needed to stretch the polymer ( $\sim 0.1$  pN for double-stranded and  $\sim 5$  pN for single-stranded DNA).

As double-stranded DNA is stretched to a length approaching its B-form contour length (3.4 Å per basepair), the force needed to further stretch the molecule increases sharply to several tens of pNs. Single-stranded DNA, on the



**FIGURE 1** Force-extension data of single- and double-stranded DNA (ssDNA and dsDNA). (a) At forces above the crossover point ( $\sim 6$  pN), single-stranded DNA is longer than double-stranded DNA. At  $\sim 65$  pN, the dsDNA undergoes a reversible and highly cooperative transition into a form that is considerably longer than B-form DNA. The red line indicates a fit of the force-extension curves with the WLC model. Base pairing and electrostatic self-avoiding effects make the WLC model break down for single-stranded DNA at lower forces.<sup>50</sup> (b) Below the crossover point, single-stranded DNA is shorter than the duplex DNA. Enzymatically catalyzed conversions between the two states can be visualized by changes in DNA length. Small differences between the two data sets in 1a and 1b can be attributed to different buffer conditions. (Figure 1a and 1b are adapted from Refs. 37 and 35, respectively. Reproduced with permission from Macmillan Publishers, Ltd.)

other hand, is not constrained by a helical geometry and can be stretched to lengths  $\sim 70\%$  larger than the double-stranded form.<sup>53</sup> A crossover point at 6 pN defines the forces below which single-stranded DNA is shorter than the duplex form and above which it can be considerably longer. As will be discussed in greater detail in this review, these well-defined differences in length can be utilized to characterize the activity of enzymes that convert one form of DNA into the other.

At forces higher than 65 pN, double-stranded DNA undergoes a reversible and highly cooperative transition into a form that is considerably longer than B-form DNA.<sup>53,54</sup> The exact nature of this transition is not well understood. Measurements of the transition free energy associated with overstretching under a variety of biochemical conditions are consistent with DNA denaturation and unwinding, possibly indicating a melting transition of double-stranded to single-stranded DNA.<sup>55,56</sup> Alternatively, it has been suggested that overstretched DNA adopts a conformation with a distinct base-paired structure.<sup>53,54</sup>

## DNA-BINDING PROTEINS

Differences in elastic properties of the various forms of DNA can be used to probe the activity of DNA-binding proteins. Elastic properties of protein filaments on DNA can be determined by force-extension experiments and the kinetics of

binding by time-resolved force spectroscopy on individual DNA molecules. These experiments will be illustrated with two examples: RecA binding to both double- and single-stranded DNA, and helix destabilization by single-stranded DNA binding proteins.

### RecA Filament Formation on DNA

The interaction of RecA with both single-stranded and double-stranded DNA plays a crucial role in bacterial recombination and DNA repair.<sup>57–59</sup> The protein catalyzes an ATP-dependent DNA strand-exchange reaction that is the central step in the repair of double-stranded DNA breaks by homologous recombination. RecA binds cooperatively to both double- and single-stranded DNA and forms highly structured filaments that facilitate the search of an invading single-stranded DNA for sites homologous in sequence on a double-stranded target.

Single-molecule stretching experiments have been employed to characterize the kinetics of RecA-DNA binding and the elastic properties of the resultant filaments. In the presence of ATP, or a nonhydrolyzable analog, RecA polymerization causes lengthening of double-stranded DNA. The persistence length of both single- and double-stranded DNA molecules fully coated with RecA dramatically increases to several hundred nm (compared to  $\sim 50$  nm and 0.8–3 nm for naked double- and single-stranded DNA, respectively).<sup>60–62</sup> Both the length increase and stiffening are believed to play an important role in

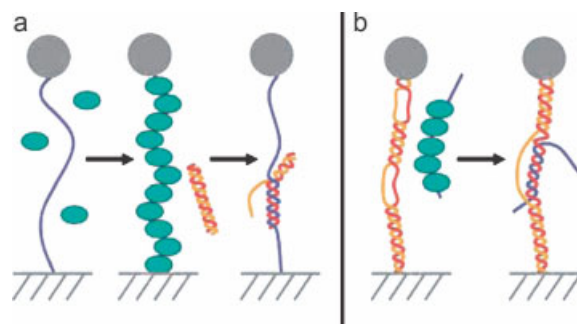
the mechanism by which RecA-DNA filaments search for homology in a target duplex molecule.

Recent work by Fulconis et al.<sup>63</sup> utilized the changes in DNA length upon RecA coating to monitor strand-exchange reactions in real time. The authors used magnetic tweezers to stretch RecA-coated single-stranded DNA and monitored the exchange with a strand of a duplex DNA molecule. Conversely, they characterized the invasion of a RecA-coated single-stranded DNA into a stretched double-stranded DNA molecule (see Figure 2). The ability to control the rotation of and measure torsion in the stretched DNA allowed the authors to demonstrate that the process of strand exchange is able to generate torque. These observations confirm the notion that during strand invasion, rapid RecA depolymerization occurs at the rear of the filament containing both the invading single-stranded DNA and target duplex. This process would relieve torsional stress, thereby assisting the front of the triple-stranded filament proceed through zones in the DNA with less than perfect homology.

### DNA Denaturation by Single-Stranded DNA Binding Proteins

Single-molecule force-extension measurements of the DNA overstretching transition have been used to study the DNA-binding properties of single-stranded DNA binding proteins (SSBs). SSBs are essential cofactors and regulators in a large number of processes involving DNA such as replication<sup>64</sup> and recombination.<sup>65</sup> Through their interaction with single-stranded DNA they prevent the formation of secondary structure and protect the DNA from nucleolytic degradation. The development of various techniques to stretch individual DNA molecules with a large range of forces allowed for the characterization of the thermodynamic and kinetic properties of single-stranded DNA coating and double-stranded DNA destabilization by SSBs.

In particular, the cooperative transition of duplex DNA at high stretching forces is used to study the DNA-binding properties of SSBs. The exact force at which this transition occurs ( $\sim 65$  pN in physiological buffer conditions; Figure 1a) is thermodynamically analogous to the melting temperature of double-stranded DNA. The influence of SSBs on this melting force provides information of the binding affinity of the protein for single-stranded DNA. In contrast to temperature-induced melting, these force studies can be performed at physiological temperatures, thus avoiding protein denaturation. Pant et al.<sup>34,66</sup> pioneered this method to study the thermodynamic and kinetic DNA-binding properties of the T4 bacteriophage SSB, the gene 32 protein (gp32). A characterization of the dependence of the gp32 equilibrium binding constant on the ionic strength revealed that protein binding



**FIGURE 2** Schematic depiction of single-molecule detection of RecA-mediated strand invasion. (a) Stretching single-stranded DNA allows for characterization of the kinetics of RecA filament formation on single-stranded DNA. Strand invasion is observed by addition of double-stranded DNA in solution. (b) Attaching all four termini of double-stranded DNA to a bead on one end and the surface on the other, allows for the introduction of torque in the duplex. The effects of RecA-mediated strand invasion can be studied by adding RecA-coated single-stranded DNA in solution. (Reproduced from Ref. 63, with permission from Macmillan Publishers, Ltd.)

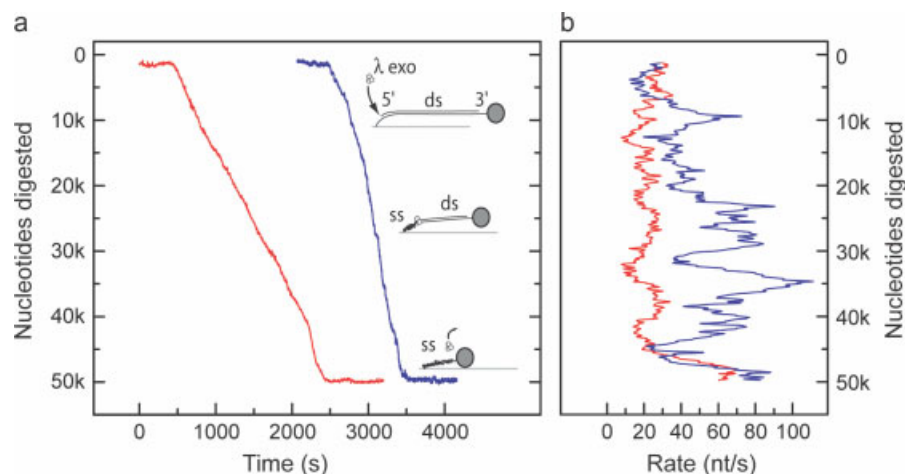
is regulated by intramolecular conformational changes (For more information, see the review by McCauley and Williams in this issue).<sup>55</sup>

## NUCLEIC-ACID ENZYMES

### Conversion Between Single- and Double-Stranded DNA

The coiling of single-stranded DNA causes it to be shorter than double-stranded DNA at low stretching forces ( $< 6$  pN; see Figure 1). At higher forces, single-stranded DNA is in a more extended conformation than the double-stranded form. Consequently, any conversion between double- and single-stranded DNA can be monitored through a change in total DNA length at constant force. The number of nucleotides converted can be calculated using the difference between the lengths of ssDNA and dsDNA at the stretching force used. The total length of a molecule containing both double-stranded and single-stranded DNA can be described as a linear combination of the lengths of the single-stranded and double-stranded regions:  $L_{\text{tot}}(F) = N_{\text{ds}}l_{\text{ds}}(F) + N_{\text{ss}}l_{\text{ss}}(F)$ , where  $L_{\text{tot}}(F)$  denotes the total extension measured at a certain force  $F$ ,  $N_{\text{ds(ss)}}$  the number of nucleotides in double-stranded (single-stranded) form, and  $l_{\text{ds(ss)}}(F)$  the length per nucleotide of double-stranded (single-stranded) DNA at force  $F$ . The sum of  $N_{\text{ds}}$  and  $N_{\text{ss}}$  is a constant and reflects the total number of nucleotides in the DNA. A measured length change  $\Delta L(F)$  then corresponds to  $\Delta N(l_{\text{ss}}(F) - l_{\text{ds}}(F))$  and directly reflects the number of double-stranded basepairs





**FIGURE 3** Single-molecule observation of  $\lambda$  exonuclease-catalyzed digestion of DNA. (a) Two single-molecule traces displaying complete enzymatic digestion of one strand of the 48.5-kb long duplex substrate. Enzymatic conversion of double-stranded into single-stranded DNA was observed as a shortening of the DNA (inset). (b) Time derivatives of the two traces in (a) as a function of enzyme position on the substrate DNA (Figure adapted from Ref. 41).

converted to single-stranded nucleotides (or  $\Delta N(l_{ds}(F) - l_{ss}(F))$  for the reverse process).

This straightforward method of observing conversions between single- and double-stranded DNA has been successfully applied to the study of DNA polymerases,<sup>36,37</sup> nucleases,<sup>41</sup> and, most recently, an entire DNA replication complex.<sup>35</sup> In the remainder of this review, these experiments will be discussed in more detail.

### Digestion of DNA by Exonucleases

Several classes of nucleases involved in repair and recombination processively degrade duplex DNA in a unidirectional fashion to convert it into single-stranded DNA. Van Oijen et al.<sup>41</sup> utilized the ability to observe changes in DNA length upon conversion from the double-stranded into the single-stranded form to study the enzyme  $\lambda$  exonuclease.  $\lambda$  exonuclease is required for recombination in bacteriophage  $\lambda$  and degrades one strand of duplex DNA in the 5' to 3' direction in a highly processive manner.<sup>67</sup> The single-stranded DNA produced by  $\lambda$  exonuclease is a substrate for homologous pairing proteins in homologous recombination.<sup>68</sup> The crystal structure of  $\lambda$  exonuclease revealed a toroidal homotrimer that presumably encircles the DNA substrate.<sup>69</sup> The free energy released during hydrolysis of the 5' penultimate nucleotide of the target strand in the substrate DNA drives the movement of the protein along the DNA. As a consequence, the enzyme requires no cofactor other than magnesium.

The effect of exonuclease activity on DNA length was characterized by attaching individual  $\lambda$  phage DNA mole-

cules on one end to the glass surface of a microfluidic flow cell and on the other end to a polystyrene bead. The DNAs were flow-stretched and their lengths monitored by tracking of the bead positions. The absolute force exerted on the beads was obtained by a fitting of the force-dependent DNA length to the worm-like chain model.<sup>39</sup> Enzymatic conversion of double- to single-stranded DNA was observed as a shortening of the DNA. To allow the protein to load onto only one of the two 5' termini, the DNA was tethered to the surface with a 3' end and to the bead with the 5' terminus of the opposite end. Since  $\lambda$  exonuclease is a stable trimer in solution, this configuration requires the protein to transiently open before it can bind the accessible 3' terminus and encircle the surface-attached 5' strand (see inset Figure 3a), resulting in extremely low binding rates. The ability to observe multiple DNA tethers simultaneously by combining the flow-stretching with wide-field microscopy allowed the observation of the rare instances of the protein loading on the DNA.

The authors stretched the DNA with a force of 2 pN, a force at which the length difference between single- and double-stranded DNA ( $l_{ds}(F) - l_{ss}(F)$ ) was maximal (Figure 1b). The change in force,  $\Delta F$ , required to change the extension of the DNA,  $\partial F/\partial L$ , was also maximized at 2 pN for both single- and double-stranded DNA. The amplitude of the Brownian motion of the tethered bead in the direction of the DNA (and thus the intrinsic uncertainty of the length measurement) is governed by the relation  $\langle \Delta L^2 \rangle = k_B T / (\partial F/\partial L)$ .<sup>44</sup> Choosing the stretching force that corresponded to the largest length changes and the smallest longitudinal noise

resulted in an optimized signal-to-noise that allowed for conversions of as little as 50 basepairs to be observed.

Once digestion of DNA molecules was started, the authors flushed the chamber with buffer lacking  $\lambda$  exonuclease. Continued shortening in cases where digestion had started proved that single enzyme molecules were responsible for the processive digestion of DNA instead of multiple enzymes undergoing repetitive cycles of binding to the DNA from solution, digestion of a small number of nucleotides, and subsequent dissociation. The high processivity of  $\lambda$  exonuclease resulted in a number of events in which a single enzyme digested one complete strand of the phage DNA (48.5 kb). Figure 3a shows two of these trajectories, with the digestion rates as a function of template position depicted in Figure 3b. The digestion rate exhibited large fluctuations during the processive degradation of dsDNA. The authors attributed these fluctuations to two sources: a sequence-dependent component that has its origin in the sequence heterogeneity of the DNA template and a component that is caused by seemingly stochastic fluctuations in the enzymatic rate.

**Sequence-Dependent Digestion Rate.** The authors measured the enzymatic rate as a function of the position of the enzyme on the  $\lambda$  phage template DNA and averaged multiple of these single-molecule rate trajectories to remove the contribution of any stochastic fluctuations in rate. Even though the measurements of many molecules were combined, the authors used the starting times of the individual digestions to synchronize the traces such that the rate fluctuations were averaged as a well-defined function of sequence. This averaging method allowed the authors to obtain sequence-dependent information that would otherwise be obscured in bulk-phase experiments.

The enzymatic digestion cycle consists of hydrolytic cleavage of a phosphodiester bond in DNA, translocation along the DNA, and melting of the 5' terminal base from neighboring bases. Hydrolysis of the phosphodiester bond is thermodynamically favorable, with a free energy change of  $-5.3$  kcal/mol in the absence of enzyme,<sup>70</sup> and is largely independent of the identity of the nucleotides. In contrast, the free energy change required to melt the 5' terminal base contains two sequence-dependent contributions: (1) the hydrogen-bonding free energy of a Watson–Crick base pair, which is different for an AT pair (two hydrogen bonds) versus a CG pair (three hydrogen bonds), and (2) the free energy of the base-stacking interaction between the 5' terminal base and its neighboring base in the same strand, which depends on the identity of both bases.

The authors simulated sequence-dependent modulations in the free-energy landscape encountered by the enzyme by

combining published free-energy values of hydrogen bonding and base stacking<sup>71,72</sup> with the known sequence of the  $\lambda$  phage DNA. By assuming that small modulations in the free energy of reaction would result in highly correlated changes in the transition energy, this thermodynamic information could be related to single-molecule kinetics. A strong correlation between the averaged rate trajectories and the predicted ones revealed that the melting of the penultimate 5' nucleotide represents a rate-limiting step in the enzymatic reaction. The folding of the single-stranded product strand into secondary or tertiary structure was ruled out as a cause of the observed sequence-dependent digestion rate by evaluating the correlation between the measured rate fluctuations and the calculated modulations in the free energy of secondary and tertiary structure formation along the ssDNA product strand.

**Stochastic Rate Fluctuations.** A large component of the fluctuations in each of the single-molecule digestion rate traces displayed no correlation with the DNA sequence. These sequence-independent fluctuations were analyzed by calculating the autocorrelation function of  $\Delta k$ ,  $C(\tau) = \langle \Delta k(0)\Delta k(\tau) \rangle - \langle \Delta k \rangle^2$ , where the rate change  $\Delta k$  is evaluated as a function of time  $\tau$  instead of nucleotide position. The timescale of the decay in the autocorrelation  $C(\tau)$  directly indicates the timescale of fluctuations in the enzymatic rate.<sup>16</sup> The nonexponential nature of the autocorrelation suggested multiple time scales in the rate fluctuations. The presence of large fluctuations in catalytic rate at a time scale comparable to or longer than the enzymatic cycle, also called dynamic disorder, has also been observed in other systems<sup>16</sup> and is thought to be associated with many long-lived conformers undergoing interconversion over a broad range of timescales.<sup>73,74</sup>

## Synthesis and Digestion of DNA by Proofreading DNA Polymerases

The synthesis of a new DNA strand on a single-stranded DNA template is catalyzed by DNA polymerases.<sup>64</sup> Both prokaryotic and eukaryotic cells contain multiple DNA polymerase activities involved in DNA replication, recombination, and repair. DNA polymerases extend a DNA chain in the 5'-3' direction by adding complementary nucleotides one at a time via 3'-hydroxyl attack of the incoming nucleotide's alpha phosphate. Most known DNA polymerases perform this reaction in a processive manner, incorporating thousands of nucleotides, without dissociating from its template. Bacterial DNA polymerases also possess a 3'-5' exonucleolytic activity that proceeds in a direction opposite to that of DNA

synthesis. The exonucleolytic removal of a mismatched penultimate nucleotide provides the capability of proofreading.

Several kinetic and structural studies have demonstrated that the rate, processivity, and efficiency of proofreading all strongly depend on subtle conformational variations within the enzyme's active site.<sup>75,76</sup> The ability to observe a single DNA polymerase moving along DNA while incorporating nucleotides allows for a more extensive study of the relation between enzyme-template interaction and kinetics. Analogous to the previously described exonuclease experiments, the rate of a single DNA polymerase synthesizing DNA on a single-strand DNA template can be measured by utilizing the difference between the elastic properties of single-stranded and double-stranded DNA.<sup>36,37</sup> By means of optical or magnetic tweezers, a force can be exerted on the DNA template and its influence on the polymerization rate investigated. For several prokaryotic polymerases, it has been demonstrated that forces higher than 30–40 pN stall the enzyme.<sup>36,37</sup> Single-molecule studies on the DNA polymerase of T7 bacteriophage showed that even higher forces stimulate the exonuclease activity by several orders of magnitude.<sup>37</sup> The response of the enzyme's translocation rates to a variation of the load applied provided information on which biochemical steps in the pathway are coupled to movement. The sensitivity of the polymerization rate to tension indicated that the rate-limiting step is directly affected by force. This behavior has been explained by a change in the conformation of the enzyme during the rate-limiting step.<sup>75</sup> A quantitative interpretation of the force-dependence<sup>36,37</sup> combined with modeling studies<sup>77</sup> revealed that this conformational change may be related to a transient interaction of the enzyme with multiple bases of the single-stranded DNA.

## DNA REPLICATION

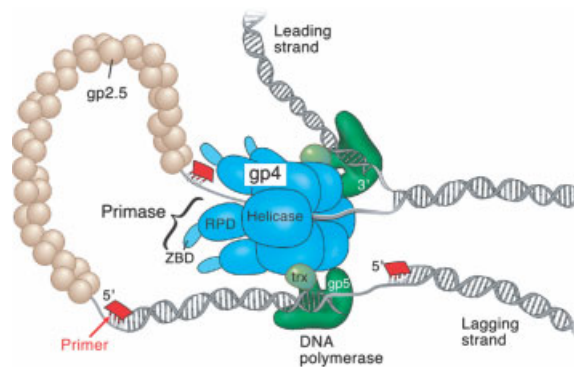
DNA replication involves the coordinated activity of a large number of proteins. The replisome, the molecular machinery of DNA replication, unwinds the double-stranded DNA, provides primers to initiate synthesis, and polymerizes nucleotides onto each of the two growing strands. Remarkable progress has been made in characterizing the structural and functional properties of the individual components; their coordination at the replication fork is less well understood. A recent single-molecule study by Lee et al.<sup>35</sup> provides new insights into how the different enzymatic activities at the replication fork are coordinated.

The general mechanism of DNA replication is highly conserved between organisms.<sup>78,79</sup> The helicase unwinds the parental double-stranded DNA (dsDNA) into two DNA strands allowing two DNA polymerases, complexed with processivity factors, to each synthesize DNA on the two single-stranded

templates. The 5' to 3' direction of polymerase-dependent nucleic-acid synthesis permits one of these enzymes to synthesize DNA in a continuous fashion on the leading strand. The opposite orientation of the lagging strand forces the second polymerase to restart at short intervals, using short RNA primers made by a DNA primase. The discontinuous synthesis of DNA on the lagging strand gives rise to a succession of Okazaki fragments that are later processed and ligated into one continuous strand. Single-stranded DNA-binding proteins remove any secondary structure that may inhibit synthesis and protect the stretches of transiently exposed ssDNA from nucleolytic attacks.

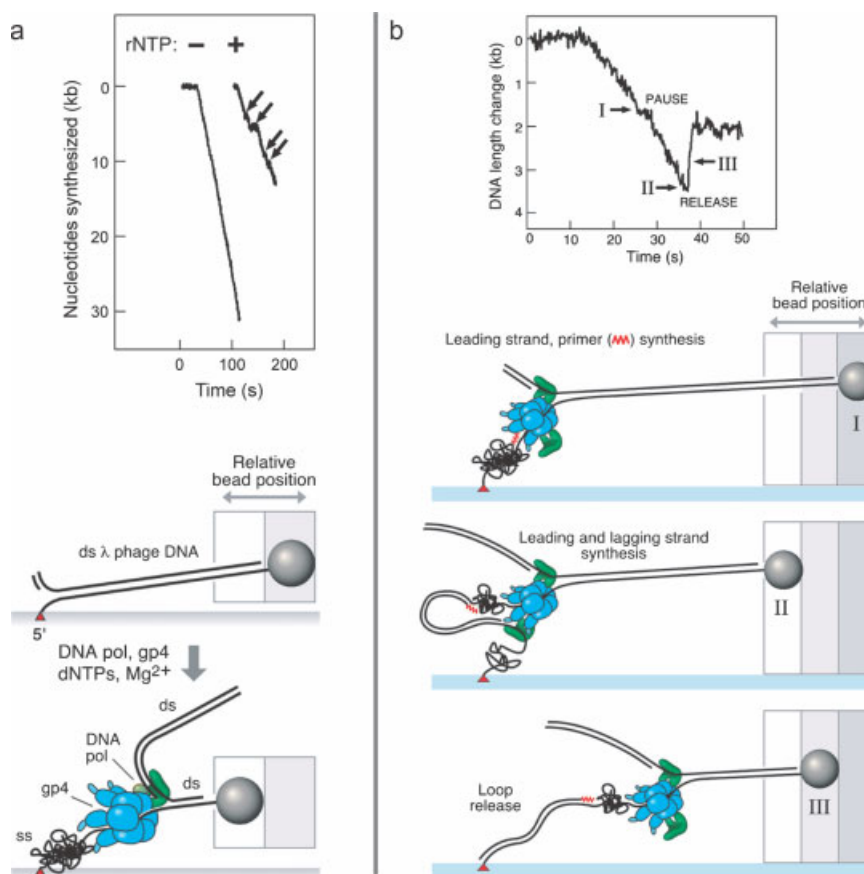
The production of an Okazaki fragment requires a series of enzymatic steps consisting of the synthesis of a primer, the recycling of the lagging-strand DNA polymerase, and the actual synthesis of the fragment. These steps must be completed sufficiently rapidly to allow the lagging-strand synthesis to remain in step with the continuous leading-strand synthesis. Different models have been put forward to explain how these slow enzymatic steps can take place at the lagging strand without losing coordination with the continuous and rapid leading-strand synthesis.<sup>80–82</sup>

Lee et al. used single-molecule techniques to directly interrogate the kinetics of leading- and lagging-strand synthesis.<sup>35</sup> The authors used the replication machinery of the T7 bacteriophage, a system that can be reconstituted *in vitro* with a small number of purified proteins (see Figure 4). A central player in T7 DNA replication is the T7 gene 4 protein (gp4), a monohexamer encircling single-stranded DNA that provides both helicase and primase activities.<sup>83,84</sup> Two copies



**FIGURE 4** The bacteriophage T7 replication machinery. The replisome consists of the hexameric T7 gene 4 protein (gp4) and two copies of the T7 DNA polymerase (T7 gene 5 protein (gp5) complexed with *E. coli* thioredoxin (trx)). T7 gene 2.5 protein (gp2.5), the ssDNA-binding protein, coats the transiently exposed ssDNA in the replication loop. Gp4 consists of a primase and a helicase domain, connected by a linker region. The primase domain consists of two subdomains: a zinc-binding domain (ZBD) and the RNA polymerase domain (RPD) (Figure reproduced from Ref. 35).





**FIGURE 5** Single-molecule observation of DNA replication. (a) Single-molecule trajectories of leading-strand synthesis (top panel). Above the traces is indicated whether ribonucleotides were present in the reaction mixture, without which no primase activity can occur. The arrows denote pauses. The bottom panel shows a schematic depiction of events observed in top panel. In the absence of lagging-strand synthesis, leading-strand synthesis causes the 5' tail of the DNA to be converted to the single-stranded form. Attachment of the 5' end to the surface allows the monitoring of this conversion as a change in total length of the DNA. (b) Single-molecule trajectory of replication loop formation. In the presence of excess T7 DNA polymerase, lagging-strand synthesis is initiated after primer synthesis (indicated by the pause) and a replication loop is formed in the lagging strand. Replication loop release is clearly visible as instantaneous lengthening of DNA. The lower panels provide a schematic explanation of these events (Figure adapted from Ref. 35).

of the T7 DNA polymerase associate with the gp4 hexamer and catalyze leading- and lagging-strand synthesis.

The authors flow-stretched the individual DNA molecules at low force (1–3 pN) and measured their shortening to probe conversion from double- to single-stranded DNA. By using a forked DNA template, a complex of one T7 DNA polymerase and hexameric gp4 could be assembled on one end of the DNA molecule. The protein complexes were assembled on the forked DNA in the absence of Mg<sup>2+</sup>. Before the reaction was initiated with Mg<sup>2+</sup>, the flow cell was stringently washed with only buffer and nucleotides, effectively removing all free protein from the flow cell and preventing protein exchange between the solution and the DNA-bound complex. This situation, difficult to achieve

using bulk biochemical techniques, allowed for a determination of the true processivity and rate of the replication complex.

Leading-strand synthesis catalyzed by T7 DNA polymerase converts one DNA strand arising from gp4 helicase activity into double-stranded DNA. In the absence of the lagging-strand DNA polymerase, the lagging strand remains in the single-stranded form. By attaching the DNA to the surface of the flow cell by the 5' (lagging) strand, leading-strand synthesis could be detected as an effective shortening of the DNA (Figure 5, trace labeled with “-rNTP”). Even though the primase domain of the gp4 is present at the replication fork, the absence of ribonucleotides (rNTP) prevents the primase from synthesizing an RNA primer.

The authors studied the primase activity of the gp4 by addition of rNTP to the reaction mixture. Short pauses were observed in the single-molecule leading-strand synthesis traces (Figure 5, trace labeled with “+rNTP”; pauses are indicated by arrows) that were demonstrated to result from primer synthesis. The observation that leading-strand synthesis momentarily stops during the production of a primer explains how the slow enzymatic events on the lagging strand take place without leading-strand synthesis progressing too far ahead of lagging-strand synthesis.

Lagging-strand synthesis was initiated by introducing T7 DNA polymerase into the flow cell during the leading-strand synthesis. The affinity for the lagging-strand polymerase to the helicase is weak in the absence of a primer-template, but the production of an RNA primer ‘locks’ the DNA polymerase into a stable binding mode to the helicase. The ensuing lagging-strand synthesis leads to the formation and release of a replication loop on the lagging strand, expressed in the single-molecule traces as a shortening of the DNA that is followed by an instantaneous lengthening (Figure 5b). The pause prior to the initiation of replication loop formation suggests that a transient halting of the whole replication fork during primase activity is required to preserve coupling.

## CONCLUSIONS

The single-molecule experiments reviewed here demonstrate how changes in DNA length can be used to obtain real-time information on turnover dynamics of nucleic-acid enzymes and provide insight into their catalytic mechanisms. The accessibility of DNA polymer properties as an observable for activity of nucleic-acid enzymes at the single-molecule level obviates the need for biochemical modification of enzymes to fluorescently tag them or allow observation otherwise.

It is the understanding of an enzyme’s functioning in its biological context that will further our knowledge of the molecular mechanisms underlying the important processes of life. The ability of these single-molecule techniques to probe not only the actions of individual enzymes but also their interactions within larger, multienzyme complexes defines an important future direction. The many conversions between DNA forms and conformers involved in both DNA replication and recombination suggest that the tools described here will become a powerful method to study these processes and will contribute significantly to our understanding of them.

The author thank the members of his research group for many insightful discussions and Anna Kochaniak for a critical reading of the manuscript.

## REFERENCES

1. Coppin, C. M.; Finer, J. T.; Spudich, J. A.; Vale, R. D. *Proc Natl Acad Sci USA* 1996, 93, 1913–1917.
2. Kuo, S. C.; Sheetz, M. P. *Science* 1993, 260, 232–234.
3. Kural, C.; Kim, H.; Syed, S.; Goshima, G.; Gelfand, V. I.; Selvin, P. R. *Science* 2005, 308, 1469–1472.
4. Svoboda, K.; Schmidt, C. F.; Schnapp, B. J.; Block, S. M. *Nature* 1993, 365, 721–727.
5. Visscher, K.; Schnitzer, M. J.; Block, S. M. *Nature* 1999, 400, 184–189.
6. Yildiz, A.; Tomishige, M.; Vale, R. D.; Selvin, P. R. *Science* 2004, 303, 676–678.
7. Funatsu, T.; Harada, Y.; Tokunaga, M.; Saito, K.; Yanagida, T. *Nature* 1995, 374, 555–559.
8. Yildiz, A.; Forkey, J. N.; McKinney, S. A.; Ha, T.; Goldman, Y. E.; Selvin, P. R. *Science* 2003, 300, 2061–2065.
9. Veigel, C.; Coluccio, L. M.; Jontes, J. D.; Sparrow, J. C.; Milligan, R. A.; Molloy, J. E. *Nature* 1999, 398, 530–533.
10. Molloy, J. E.; Burns, J. E.; Kendrick-Jones, J.; Tregear, R. T.; White, D. C. *Nature* 1995, 378, 209–212.
11. Finer, J. T.; Simmons, R. M.; Spudich, J. A. *Nature (London)* 1994, 368, 113–119.
12. Hille, B. *Ion Channels of Excitable Membranes*; Sinauer: Sunderland, MA, 2001.
13. Capaldi, R. A.; Aggeler, R. *Trends Biochem Sci* 2002, 27, 154–160.
14. Yasuda, R.; Noji, H.; Yoshida, M.; Kinoshita, K., Jr.; Itoh, H. *Nature (London)* 2001, 410, 898–904.
15. Kaim, G.; Prummer, M.; Sick, B.; Zumofen, G.; Renn, A.; Wild, U. P.; Dimroth, P. *FEBS Lett* 2002, 525, 156–163.
16. Lu, H. P.; Xun, L.; Xie, X. S. *Science* 1998, 282, 1877–1882.
17. Bustamante, C.; Macosko, J. C.; Wuite, G. J. L. *Nat Rev Mol Cell Biol* 2000, 1, 130–136.
18. Herbert, K. M.; La Porta, A.; Wong, B. J.; Mooney, R. A.; Neuman, K. C.; Landick, R.; Block, S. M. *Cell* 2006, 125, 1083–1094.
19. Abbondanzieri, E. A.; Greenleaf, W. J.; Shaevitz, J. W.; Landick, R.; Block, S. M. *Nature* 2005, 438, 460–465.
20. Shaevitz, J. W.; Abbondanzieri, E. A.; Landick, R.; Block, S. M. *Nature* 2003, 426, 684–687.
21. Neuman, K. C.; Abbondanzieri, E. A.; Landick, R.; Gelles, J.; Block, S. M. *Cell* 2003, 115, 437–447.
22. Yin, H.; Wang, M. D.; Svoboda, K.; Landick, R.; Block, S. M.; Gelles, J. *Science* 1995, 270, 1653–1657.
23. Schafer, D. A.; Gelles, J.; Sheetz, M. P.; Landick, R. *Nature* 1991, 352, 444–448.
24. Joo, C.; McKinney, S. A.; Nakamura, M.; Rasnik, I.; Myong, S.; Ha, T. *Cell* 2006, 126, 515–527.
25. van Mameren, J.; Modesti, M.; Kanaar, R.; Wyman, C.; Wuite, G. J.; Peterman, E. J. *Biophys J* 2006, 91, L78–L80.
26. Graneli, A.; Yeykal, C. C.; Robertson, R. B.; Greene, E. C. *Proc Natl Acad Sci USA* 2006, 103, 1221–1226.
27. Spies, M.; Bianco, P. R.; Dillingham, M. S.; Handa, N.; Baskin, R. J.; Kowalczykowski, S. C. *Cell* 2003, 114, 647–654.
28. Handa, N.; Bianco, P. R.; Baskin, R. J.; Kowalczykowski, S. C. *Mol Cell* 2005, 17, 745–750.
29. Amitani, I.; Baskin, R. J.; Kowalczykowski, S. C. *Mol Cell* 2006, 23, 143–148.

30. Bianco, P. R.; Brewer, L. R.; Corzett, M.; Balhorn, R.; Yeh, Y.; Kowalczykowski, S. C.; Baskin, R. J. *Nature (London)* 2001, 409, 374–378.
31. Blainey, P. C.; van Oijen, A. M.; Banerjee, A.; Verdine, G. L.; Xie, X. S. *Proc Natl Acad Sci USA* 2006, 103, 5752–5757.
32. Zhang, Z.; Spiering, M. M.; Trakselis, M. A.; Ishmael, F. T.; Xi, J.; Benkovic, S. J.; Hammes, G. G. *Proc Natl Acad Sci USA* 2005, 102, 3254–3259.
33. Myong, S.; Rasnik, I.; Joo, C.; Lohman, T. M.; Ha, T. *Nature* 2005, 437, 1321–1325.
34. Pant, K.; Karpel, R. L.; Rouzina, I.; Williams, M. C. *J Mol Biol* 2004, 336, 851–870.
35. Lee, J.-B.; Hite, R. K.; Hamdan, S. M.; Xie, X. S.; Richardson, C. C.; van Oijen, A. M. *Nature* 2006, 439, 621–624.
36. Maier, B.; Bensimon, D.; Croquette, V. *Proc Natl Acad Sci USA* 2000, 97, 12002–12007.
37. Wuite, G. J. L.; Smith, S. B.; Young, M.; Keller, D.; Bustamante, C. *Nature (London)* 2000, 404, 103–106.
38. Bustamante, C.; Bryant, Z.; Smith, S. B. *Nature (London)* 2003, 421, 423–427.
39. Bustamante, C.; Marko, J. F.; Siggia, E. D.; Smith, S. *Science* 1994, 265, 1599–1600.
40. Marko, J. F.; Siggia, E. D. *Macromolecules* 1994, 27, 981–988.
41. van Oijen, A. M.; Blainey, P. C.; Crampton, D. J.; Richardson, C. C.; Ellenberger, T.; Xie, X. S. *Science* 2003, 301, 1235–1239.
42. Wuite, G. J. L.; Davenport, R. J.; Rappaport, A.; Bustamante, C. *Biophys J* 2000, 79, 1155–1167.
43. Smith, S. B.; Finzi, L.; Bustamante, C. *Science* 1992, 258, 1122–1126.
44. Strick, T. R.; Allemand, J. F.; Bensimon, D.; Bensimon, A.; Croquette, V. *Science* 1996, 271, 1835–1837.
45. Strick, T. R.; Croquette, V.; Bensimon, D. *Nature (London)* 2000, 404, 901–904.
46. Strick, T. R.; Allemand, J. F.; Bensimon, D.; Croquette, V. *Biophys J* 1998, 74, 2016–2028.
47. Ashkin, A.; Dziedzic, J. M. *Science* 1987, 235, 1517–1520.
48. Ashkin, A. *Proc Natl Acad Sci USA* 1997, 94, 4853–4860.
49. Bustamante, C.; Chemla, Y. R.; Forde, N. R.; Izhaky, D. *Annu Rev Biochem* 2004, 73, 705–748.
50. Dessinges, M. N.; Maier, B.; Zhang, Y.; Peliti, M.; Bensimon, D.; Croquette, V. *Phys Rev Lett* 2002, 89, 248102.
51. Lu, Y.; Weers, B.; Stellwagen, N. C. *Biopolymers* 2001, 61, 261–275.
52. Mills, J. B.; Vacano, E.; Hagerman, P. J. *J Mol Biol* 1999, 285, 245–257.
53. Smith, S. B.; Cui, Y.; Bustamante, C. *Science* 1996, 271, 795–799.
54. Cluzel, P.; Lebrun, A.; Heller, C.; Lavery, R.; Viovy, J.-L.; Chate-nay, D.; Caron, F. *Science* 1996, 271, 792–794.
55. McCauley, M. J.; Williams, M. C. *Biopolymers* 2007, 85, 154–168.
56. Williams, M. C.; Rouzina, I.; Bloomfield, V. A. *Acc Chem Res* 2002, 35, 159–166.
57. Eggleston, A. K.; West, S. C. *Trends Genet* 1996, 12, 20–26.
58. Bell, C. E. *Mol Microbiol* 2005, 58, 358–366.
59. Prevost, C.; Takahashi, M. Q. *Rev Biophys* 2003, 36, 429–453.
60. Hegner, M.; Smith, S. B.; Bustamante, C. *Proc Natl Acad Sci USA* 1999, 96, 10109–10114.
61. Bennink, M. L.; Scharer, O. D.; Kanaar, R.; Sakata-Sogawa, K.; Schins, J. M.; Kanger, J. S.; De Grooth, B. G.; Greve, J. *Cytometry* 1999, 36, 200–208.
62. Shivashankar, G. V.; Feingold, M.; Krichevsky, O.; Libchaber, A. *Proc Natl Acad Sci USA* 1999, 96, 7916–7921.
63. Fulconis, R.; Mine, J.; Bancaud, A.; Dutreix, M.; Viovy, J. L. *EMBO J* 2006, 25, 4293–4304.
64. Kornberg, A.; Baker, T. A. *DNA Replication*; W. H. Freeman: New York, 1992.
65. Kowalczykowski, S. C. *Trends Biochem Sci* 2000, 25, 156–165.
66. Pant, K.; Karpel, R. L.; Williams, M. C. *J Mol Biol* 2003, 327, 571–578.
67. Subramanian, K.; Rutvisuttinunt, W.; Scott, W.; Myers, R. S. *Nucleic Acids Res* 2003, 31, 1585–1596.
68. Cassuto, E.; Radding, C. M. *Nature New Biol (London)* 1971, 229, 13–16.
69. Kovall, R.; Matthews, B. W. *Science* 1997, 277, 1824–1827.
70. Dickson, K. S.; Burns, C. M.; Richardson, J. P. *J Biol Chem* 2000, 275, 15828–15831.
71. Bommarito, S.; Peyret, N.; SantaLucia, J., Jr. *Nucleic Acids Res* 2000, 28, 1929–1934.
72. Kool, E. T. *Annu Rev Biophys Biomol Struct* 2001, 30, 1–22.
73. Frauenfelder, H.; Sligar, S. G.; Wolynes, P. G. *Science* 1991, 254, 1598–1603.
74. Xie, X. S. *J Chem Phys* 2002, 117, 11024–11032.
75. Wong, I.; Patel, S. S.; Johnson, K. A. *Biochemistry* 1991, 30, 526–537.
76. Doublet, S.; Tabor, S.; Long, A. M.; Richardson, C. C.; Ellenberger, T. *Nature (London)* 1998, 391, 251–258.
77. Goel, A.; Frank-Kamenetskii, M. D.; Ellenberger, T.; Hersch-bach, D. *Proc Natl Acad Sci USA* 2001, 98, 8485–8489.
78. Waga, S.; Stillman, B. *Annu Rev Biochem* 1998, 67, 721–751.
79. Benkovic, S. J.; Valentine, A. M.; Salinas, F. *Annu Rev Biochem* 2001, 70, 181–208.
80. Salinas, F.; Benkovic, S. J. *Proc Natl Acad Sci USA* 2000, 97, 7196–7201.
81. Tougu, K.; Marians, K. J. *J Biol Chem* 1996, 271, 21398–21405.
82. Lee, J.; Chastain, P. D., II; Griffith, J. D.; Richardson, C. C. *J Mol Biol* 2002, 316, 19–34.
83. Frick, D. N.; Baradaran, K.; Richardson, C. C. *Proc Natl Acad Sci USA* 1998, 95, 7957–7962.
84. Guo, S.; Tabor, S.; Richardson, C. C. *J Biol Chem* 1999, 274, 30303–30309.

*Reviewing Editor: Nils Walter*